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We previously demonstrated that the human reovirus preferentially infects cancer cells with an activated Ras signalling pathway. In this study, we investigated the feasibility of reovirus as a therapeutic against breast cancer. We examined a panel of breast cancer cell lines as well as a non-tumorigenic epithelial cell line derived from normal tissue for *in vitro* reovirus replication. All five of the tumor-derived cell lines were efficiently lysed by the virus, while the non-transformed cells were resistant to infection. One of the cell lines, MDA-MB-435S, was implanted in the mammary fat pad of SCID mice as xenografts, which were subsequently injected with reovirus. Tumor regression was found to be rapid and complete. Furthermore, in a bilateral tumor SCID mouse model, a single unilaterally injection with reovirus resulted in dramatic regression of the tumor size in both the injected and remote tumors, raising the therapeutic possibility of systemic delivery of reovirus. Finally, the ability of reovirus to act against *primary* breast cancer tumors was determined *ex vivo*; we found that reovirus could effectively replicate in primary human breast tumor specimens.

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Introduction

We have previously demonstrated that the human reovirus specifically infects (and therefore kills) cancer cells with an activated Ras pathway. Injection of reovirus into human glioblastoma xenografts in a SCID mouse model resulted in dramatic tumor regression. The purpose of the present study was to test the potential use of reovirus as an anti-breast cancer therapeutic. The scope of the research will encompass both *in vitro* and *in vivo* studies using human breast cancer cell lines, primary breast tumors, as well as xenografts in a SCID mouse model.

BODY

Title: The Use of Reovirus as an Anti-Breast Cancer Agent

In the approved statement of work, five tasks were listed. Of the five, we have almost finished the first two. These are:

Task 1. To test the susceptibility of various breast cancer cell lines and primary breast cancer tissues to reovirus treatment *in vitro* (months 1-9).

Task 2. To test the effect of reovirus treatment on breast cancer xenografts in SCID mice (months 10-15).

Our research accomplishments are summarized in a paper (in preparation) appended below.

Pertaining to task 1, we have examined a panel of breast cancer cell lines as well as a non-tumorigenic epithelial cell line derived from normal tissue for *in vitro* reovirus replication. We found that all five of the tumor-derived cell lines were efficiently lysed by the virus, while the non-transformed cells were resistant to infection. We have also examined the ability of reovirus to act against primary breast cancer tumors (*ex vivo* samples), and found that reovirus could effectively replicate in these specimens.

Pertaining to task 2, we injected reovirus into MDA-MB-435S mammary fat pad tumor xenografts in SCID mice, and found that tumor regression was rapid and complete from a single injection of reovirus. We also used a bilateral tumor model in which the virus was injected unilaterally. A single injection of reovirus resulted in dramatic reduction of the tumor size in both the injected and remote tumors, raising the therapeutic possibility of systemic delivery of reovirus.

TITLE: The Use of Reovirus as an Anti-Breast Cancer Agent

ABSTRACT: Previously, we have shown that the human reovirus that is restricted to replicate in those cells with an activated Ras pathway could be used as an effective oncolytic agent against human glioblastoma xenographs in a SCID mouse model. This study examines in more detail the feasibility of reovirus as a therapeutic against breast cancer, a subset of cancer in which direct activating mutations in the *ras* proto-oncogene are relatively rare, yet where uncoupled stimulation of the Ras pathway by upstream elements is important in the disease's pathogenesis. We examined a panel of breast cancer cell lines as well as a non-tumorigenic epithelial cell line derived from normal tissue for *in vitro* reovirus replication. All five of the tumor-derived cell lines were efficiently lysed by the virus, while the non-transformed cells were resistant to infection. To determine if reovirus could be used as an oncolytic agent against breast tumors *in vivo*, MDA-MB-435S mammary fat pad tumor xenografts were injected with reovirus or were mock-treated with UV-inactivated virus. Tumor regression was rapid and complete. Furthermore, SCID/NOD mice were implanted with MDA-MB-468 human tumor xenographs in a bilateral tumor model, and unilaterally injected with reovirus. The single injection resulted in dramatic regression of the tumor size in both the injected and remote tumors, raising the therapeutic possibility of systemic delivery of reovirus. Finally, the ability of reovirus to act against *primary* breast cancer tumors was determined *ex vivo*; we found that reovirus could effectively replicate in primary human breast tumor specimens.

INTRODUCTION

Breast cancer is one of the most common and feared cancers, with an estimated lifetime incidence rate of one in eight for American women, and with a severe societal impact in terms of number of years lost due to early death from the disease [NCI-SEER, 1998]. Although there have been many advances in the breast cancer field that have allowed for a stable mortality rate in the face of an increasing incidence rate, most of this progress can be attributed to improved early *detection* and not to novel *treatment* strategies. The fact that there have been few advances in the actual treatment of this affliction demands that the development of unconventional therapeutic strategies be explored.

Previously, we have described one such therapeutic, the double-stranded RNA reovirus, whose oncolytic capabilities and benign pathogenic profile make it an attractive potential alternative in the management of cancer [1]. Such an application for a relatively innocuous virus arose from studies establishing that reovirus could effectively replicate in and lyse only those cells that contain an activated Ras signaling pathway. Activation, leading to susceptibility to reovirus infection, could arise either through direct mutation of *ras* itself, or through activation of upstream elements [2;3]. This lead to the proposition that reovirus might be effective as an anti-cancer therapeutic, as transformation by Ras plays an important role in a large proportion of human cancers. When tested *in vivo*, reovirus did indeed demonstrate oncolytic properties, selectively targeting and causing complete regression of U87 tumor xenografts in SCID mice and Ras-activated tumor allografts in C3H mice [1].

In this study we expand the model of reovirus as an oncolytic agent to include breast tumors. Despite the fact that activating mutations in *ras* are infrequent in breast cancer [4-6], there is a growing body of evidence that activation of the Ras/ERK1/2 pathway is important in the initiation and progression of this disease. It is well established that upstream elements, which typically stimulate the Ras pathway, are frequently overexpressed in breast cancers. For instance, a member of the epidermal growth factor receptor (EGFR) family, Her-2 (Neu/ErbB-2), is amplified and overexpressed in approximately 30% of all breast cancers, and is associated with a poor patient prognosis [7;8]. This transforming ability of Her-2 is dependent upon Ras activity and leads to signalling through the ERK/MAP kinase pathway [9-11]. Additional elements upstream of Ras have also been implicated in the etiology of breast cancer. The EGFR itself has been observed to be overexpressed in breast tumors [12;13]. Moreover, Src-family non-receptor tyrosine kinases have also been found to be activated in a significant population of breast tumors [14;15].

Because activation of elements upstream of Ras is important in breast tumor pathogenesis, it follows that reovirus could prove to be a plausible alternative therapy in the management of breast cancer. To achieve this end, we demonstrate that reovirus can effectively replicate in a panel of breast cancer cell lines *in vitro*; additionally, we show that reovirus can eradicate breast tumor xenografts *in vivo* at an orthotopic site. Furthermore, we now observe that reovirus is capable of targeting and causing regression of tumors distant from the site of injection. Finally, we find that reovirus is capable of replicating in primary human tumor tissue in an *ex vivo* context. Altogether, these results indicate that reovirus could conceivably be an effective oncolytic agent against breast tumors.

RESULTS

Human breast tumor cell lines are susceptible to oncolysis by reovirus in vitro

To assess the feasibility of reovirus as an anti-breast tumor therapeutic, a panel of breast cancer cell lines was examined for susceptibility to reovirus infection. MDA-MB-468, MCF7, MDA-MB-435S, T-47D, and SK-BR-3 cells, derived from various breast tumors, were first tested *in vitro* for their capacity to support an infection by reovirus. Susceptibility to reovirus infection in these tumor-derived cell lines was compared to infection of HBL-100 cells derived from normal breast tissue.

Initially, gross morphology of cells infected by reovirus was observed (Fig. 1). The six cell lines were challenged with reovirus at a multiplicity of infection (MOI) of 10; cytopathic effect (CPE) due to reovirus infection was examined at 48 hours post-infection. Productively infected cells display a rounded, granular phenotype just prior to death induced by reovirus, whereas resistant cells retain their healthy morphology after viral challenge. All tumor-derived cell lines exhibited striking cytopathic effect, whereas cells derived from normal tissue remain unaffected by exposure to reovirus.

To further demonstrate reovirus replication in breast tumor-derived cells, the capacity of these cells to support reovirus protein synthesis and the status of host protein synthesis was investigated (Fig 2). At 22 hours post-infection, cells were metabolically labelled with ^{35}S-methionine for four hours. The results clearly demonstrate effective reovirus protein synthesis, combined with an abrogation of host protein synthesis, within all breast tumor-derived cell lines examined. In contrast, reovirus replication was restricted in the HBL-100 cell line. Immunoprecipitation of reovirus proteins further confirms that reovirus replication was limited

to the tumor-derived cell lines. Low levels of reovirus protein found in the T-47D and SK-BR-3 cells is likely due to the fact that the lytic cycle in these cells is very rapid and thus, by 26 hours, most of the cells had already been lysed.

Reovirus therapy of orthotopic human breast cancer xenografts leads to tumor regression

To determine if the susceptibility of breast tumor cell lines determined *in vitro* is predictive of reovirus effectiveness as an anti-breast cancer therapeutic *in vivo*, the human breast cancer cell line, MDA-MB-435S was used to establish tumor xenografts in SCID/NOD mice. As the orthotopic site of tumor growth may represent a more appropriate model of breast cancer, the oncolytic capacity of reovirus was assayed in this system. In this model, the cells were implanted subcutaneously over the mammary fat pad of female SCID/NOD mice. Once palpable tumors had been established, 1×10^7 plaque forming units (PFU) of either live virus or UV-inactivated reovirus was administered intratumorally, and tumor growth was followed for a period of 28 days. We found that mice treated with live virus exhibited rapid and dramatic tumor regression, whereas tumors treated with UV-inactivated virus had sustained tumor growth (Fig. 3). In figure 4, photos of representative test and control animals are shown that demonstrate clear tumor regression in the live-virus-treated animals. Although some morbidity was observed in terms of hind limb necrosis (as previously documented by Coffey *et al* (1998)), tumor regression was generally complete before reovirus pathogenesis in the SCID/NOD mice. These results demonstrate that, even in a microenvironment that promotes the natural and aggressive growth of mammary tumors, reovirus can effectively cause tumor regression.

Reovirus therapy can effect breast tumor xenograft regression* in vivo *by direct injection as well as by systemic spread

Metastasis of the primary tumor to localized and regional sites is a significant risk to patients with breast cancer, and thus it is important to consider the treatment of remote tumors when assessing the potential effectiveness of a novel therapeutic. Therefore, we further tested the ability of reovirus to not only replicate within the injected tumor, but also to spread from the place of injection and effect tumor regression at distant sites. Furthermore, to ensure that reovirus-mediated tumor regression seen in the orthotopic model was not restricted to one *in vivo* breast tumor xenograft type, the human breast carcinoma cell line, MDA-MB-468, was used. Cells were introduced subcutaneously in sites overlying both the left and right hind flanks; following establishment of palpable tumors, only the tumor overlying the left flank was administered a single intraneoplastic injection of 1.0×10^7 PFU of reovirus. Control animals were given an intratumoral injection of UV-inactivated reovirus and tumor growth was followed for a period of four weeks (Fig. 5). The reovirus treatment resulted in a dramatic regression in tumor size of the injected (left) flank, as well as the contralateral (right) site. Hematoxylin/eosin (HE) staining of the remaining mass indicated that the administration of reovirus resulted in the elimination of tumor cells at the injected site, relative to control tumors (Fig. 6, panels a and b).

To confirm that regression was due to systemic spread and tumor targeting by reovirus, immunohistochemical staining for reovirus proteins was conducted. Staining of paraffin-embedded thin sections of the remaining tumor mass determined that reovirus proteins were indeed present in the contralateral tumor (Fig.6). Proteins were localized only to the periphery of the tumor scar tissue, in necrotic areas, as well as in remaining live tumor cells, suggestive of continuing active viral replication in the cells not yet lysed by reovirus at the time of sacrifice

(Fig. 6, panel c). Reovirus replication preferentially localized to the tumor site, leaving the surrounding normal tissue architecture intact. Viral protein could not be detected in the control tumors injected with UV-inactivated virus (Fig. 6, panel d).

Primary human breast tumor biopsy tissue can support infection by reovirus

To ensure that the oncolytic effect of reovirus is not due to an innate characteristic of a passaged cell line, a primary sample of human breast cancer was obtained and assessed for reovirus infectability. Pathological examination found this first sample to be a node-negative, estrogen and progesterone receptor-negative, infiltrating ductal carcinoma with a Richardson scale grade 3 out of 3. Her-2 staining was negative (data not shown). A single cell suspension was created from this breast tumor biopsy tissue and infected with reovirus. From 24 to 48 hours post-infection, cells were labelled with [^{35}S]-methionine-containing medium and lysed. Lysates were analyzed by SDS-PAGE followed by autoradiography.

Significant reovirus protein synthesis was observed in the infected primary biopsy tissue, accompanied by a marked decrease in host protein synthesis (compared to uninfected lysate) (Fig. 7). This is characteristic of the late stages of reovirus infection in acutely susceptible cells, in which viral protein synthesis eventually eclipses host protein synthesis just prior to cell death.

DISCUSSION

Using a panel of breast cancer cell lines, we have clearly demonstrated that reovirus can replicate within all breast tumor-derived cell lines tested. This was evident by examination of both

cytopathic effect as well as viral protein synthesis during infection. The observed susceptibility could be due to one of many cellular abnormalities, although it is relatively unlikely that the susceptibility is due to activating mutations in Ras itself (MFC7, T-47D and MDA-MB-468 cells do not harbour activating mutations in codons 12 or 13 [6]).

A more likely possibility is that upstream signalling leading to Ras pathway activation is rendering these cells susceptible to reovirus oncolysis; indeed, Her-2 levels are high in MCF7 and SK-BR-3 cells compared to the uninfectable HBL-100 cell line [16]. Studies in our lab have also found that normally resistant Rat-1 cells are rendered susceptible to reovirus infection upon transformation with an activated form of Her-2 (K.L. Norman, P.W.K. Lee and W. J. Muller, unpublished observation). Together, these data indicate that reovirus may prove to be very effective in the treatment of the thirty percent of breast cancers in which the Her-2/*neu*/*erbB-2* gene is amplified or overexpressed.

However, reovirus oncolysis is not only limited to those breast cancers with Her-2 overexpression. Alternatively, activation of non-receptor tyrosine kinases may exert an influence on reovirus susceptibility in breast cancer cells. Hyper-activation of the Src family of non-receptor tyrosine kinases has also proven to be a salient feature of breast tumor tissue. A number of studies indicate a four to thirty-fold increase in Src activity in primary breast tumors when compared to normal breast tissue [14;15]. Activation of Src family kinases also leads to Ras activation; thus, this subset of breast tumors could potentially be included in the population of reovirus-sensitive cancers. We have indeed found that v-*src* transformation of NIH-3T3 cells renders them

susceptible to reovirus infection *in vitro* (data not shown). Altogether, breast tumor susceptibility to reovirus can be conferred by several mechanisms of Ras pathway activation.

When tested in murine models of breast cancer, reovirus demonstrates superior *in vivo* anti-tumor activity. Previously, we have demonstrated reovirus-mediated regression of U87 glioblastoma hind flank subcutaneous tumors in mice. However, human breast tumor xenografts have been found to grow preferentially (and often more aggressively) when implanted into orthotopic sites of immunodeficient mice [17-20]. This suggests that the microenvironment (eg. growth factors, stromal interactions) provided by the mammary fat pad might better support the growth of breast tumor xenografts [21]. Nonetheless, even at this anatomically appropriate location which fosters the aggressive growth of the tumor, reovirus is still capable of effecting complete tumor regression.

Of note, it has been previously documented that SCID/NOD mice are unable to clear reovirus infection due to their lack of an effective cellular and humoral immune response, and some animal morbidity was observed in this model as previously documented (data not shown)[1]. Interestingly, reovirus intratumoral therapy in tumor-bearing nude mice does not lead to morbidity and mortality, despite the fact that these animals also have an impaired immune response (K. Hirasawa and P.W.K. Lee, unpublished observation).

We have furthermore been able to demonstrate that reovirus is capable of targeting tumors remote from the site of injection, raising the possibility for systemic treatment of metastases.

This is especially pertinent to the therapy of breast cancer, when one considers that death due to metastasis is a significant risk for patients with this disease.

Finally, the capacity of reovirus to replicate in primary human breast tumor tissue *ex vivo* has been demonstrated. This substantiates the hypothesis that reovirus could effect killing of human tumors; we demonstrate that susceptibility to reovirus is not simply a characteristic of human tumor cell lines that have been propagated in culture, and moreover, that reovirus-mediated tumor regression in mice is not an innate quality of rodent tumor models.

As mentioned above, current shortfalls in the management of breast cancer demand the examination and development of novel therapeutics. New antibody-mediated therapies for breast cancer, currently in clinical trials, have shown success against Her-2-overexpressing tumors due to their ability to bind receptors and effect an intracellular response (eg. trastuzumab/Herceptin) [22-24]. However, as not all breast tumors respond, it remains important to find alternative modes of treatment that can substitute where novel therapeutics fail. Fortunately, it is possible that some difficulties associated with antibody-mediated therapy could be made up for by the use of reovirus. Firstly, therapeutics that are designed to target growth factor receptors such as Her-2 may be of limited value, as not all breast cancers express these extracellular components at elevated levels, and thus clearly not all will respond to receptor-directed treatment. Conversely, tumor susceptibility to reovirus should encompass all those cancers in which Ras pathway activity is elevated, regardless of the status of any individual extracellular element. We have, in fact, demonstrated reovirus replication in this paper in a primary human tumor specimen expressing undetectable levels of Her-2 (Fig. 7). While the applicability of specifically targeted

antibodies is restricted in the face of the broad range of intracellular and extracellular abnormalities found in breast cancer, the increased Ras pathway activity stemming from many of these abnormalities antithetically leads to a more extensive therapeutic application of reovirus oncolysis.

Secondly, monoclonal antibodies directed against Her-2 are not themselves cytocidal, but are rather, cytostatic [25;26]. There is, however, evidence that treatment of tumors with anti-Her-2 antibodies in an immune competent organism may actually result in a cytocidal effect mediated by antibody-dependent cellular cytotoxicity (ADCC)[26;27]. Conversely, reovirus alone is a cytocidal agent and does not rely upon immune effector cells to cause tumor regression, as demonstrated by the use of SCID/NOD mice (this paper and (1)), which have an impaired lymphocyte-mediated immune response as well as severely impaired natural killer cell function.[28]

In addition to antibodies' reliance on effector cells to mediate tumor regression, the biology of tumor blood vessels may also pose difficulties insomuch as their limited capacity to deliver these therapeutics to all target cells. It has been shown that the vessels with the greatest permeability to macromolecules reside primarily at the tumor-host interface, while the least permeable vessels are those that actually penetrate the tumor mass [29;30]. The result of this differential permeability is that tumor-specific antibodies, and notably, their cytocidal effector cells, would have difficulty penetrating into the tumor mass and thus, may only be effective at the periphery. Ultimately, reovirus replication, once initiated within a tumor (either through direct injection or *via* systemic

delivery), does not rely on blood vessel permeability for further penetration into the tumor, nor on cytocidal effector cells for mediation of cell killing and actual tumor regression.

Never in the history of cancer biology has there been a more promising period in the development of novel therapeutics for the treatment of breast cancer. Several therapeutics are targeted at specific receptors that are overexpressed in subsets of this cancer, and many are meeting with some clinical success. Perhaps one of the most promising new therapies involves the use of antibodies directed against Her-2, an appropriate treatment for approximately thirty percent of all breast cancers. Fortunately, limitations associated with the use of such antibodies may be overcome with the use of reovirus, making it an attractive candidate as an alternative cancer therapy. Reovirus should target those breast cancers in which there is activation of the Ras pathway. This specificity, however, is not restricted to activating mutations of the Ras protein itself (admittedly a rare subset of breast tumors) but also includes activation of Ras stemming from upstream elements. These elements not only encompass receptor tyrosine kinases such as EGFR and Her-2, but also include non-receptor tyrosine kinases such as those of the Src family. Importantly, all of these elements have been implicated in the etiology of breast cancer. Taken together, the evidence presented here clearly demonstrates that reovirus has the potential to be used with great efficacy as a therapy for a tumor type as heterogeneous as breast cancer.

METHODS

Cells and virus

HBL-100, MDA-MB-468, MCF7, MDA-MB-435S, T-47D, and SK-BR-3 cells were a gift from Dr. Karl Riabowol (University of Calgary). Cells were all maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum (FBS) (Gibco/BRL) and antibiotics. Unless otherwise specified, all reagents were obtained from Sigma.

The Dearing strain of reovirus serotype 3 was propagated in L929 cells grown in suspension in Joklik's modified Eagle's medium containing 5 % FBS. Virus was purified according to the protocol of Smith *et al* (1969) with the exception that β-mercaptoethanol was omitted from the extraction buffer.

Radiolabelling of reovirus-infected cells and immunoprecipitation of labeled reovirus proteins

Eighty-percent confluent monolayers of the cell lines described were infected with reovirus at a multiplicity of infection (MOI) of 10 plaque forming units per cell. After a period of 22 hours, the medium was replaced with methionine and cysteine-free Dulbecco's modified Eagle's medium (DMEM) containing 10 % dialyzed FBS and 50 μCi/ml of [^{35}S]-methionine (Amersham). After four hours incubation, the medium was removed and cells were washed with phosphate buffered saline (PBS) and lysed in PBS + 1 % Triton X-100, 0.5 % sodium deoxycholate and 1 mM EDTA. Nuclei were removed by centrifugation and supernatants were stored at – 70°C until use.

Polyclonal rabbit anti-reovirus serotype 3 serum was used for immunoprecipitation of [^{35}S]-methionine-labeled reovirus proteins from cell lysates, as previously documented by Lee *et*

al (1981). Immunoprecipitated proteins were subjected to SDS-PAGE as described by Laemmli (1970), followed by autoradiography.

Reovirus therapy of breast cancer tumor xenografts

Tumor xenografts were established and treated as described in Coffey *et al* (1998). MDA-MB-435S human breast tumor cells were grown *in vitro*, trypsinised, and 2 x 10^6 cells were implanted over a mammary fat pad of SCID/NOD mice (Cross Cancer Institute, Canada). Once palpable tumors had been established, tumors were administered 1 x 10^7 PFU of either live or UV-inactivated reovirus by direct injection and monitored for a period of 30 days.

For the bilateral breast tumor xenograft model, 1 x 10^6 MDA-MB-468 cells were implanted subcutaneously in a site overlying each hind flank of SCID/NOD mice. Once palpable tumors had been established, 1 x 10^7 PFU of live reovirus in PBS was injected into the left flank tumor, or an equivalent amount of UV-inactivated reovirus was administered to control mice tumors. Tumor growth was monitored for a period of four weeks.

Immunohistochemistry of reovirus antigens in tumor xenografts

Upon completion of timecourses, mice were perfused with PBS and then with formalyn prior to tumor excision. Tumors (or remaining masses) were fixed in formalyn and embedded in paraffin prior to sectioning and mounting on Permafrost slides. Sections were then immersed in xylene, followed by rehydration in decreasing concentrations of ethanol. Endogenous peroxidase was inactivated in 3 % hydrogen peroxide in methanol for 15 minutes. Sections were then incubated in primary rabbit anti-reovirus polyclonal antibody (1/1000 in PBS with 10 % goat serum and 0.1 % Triton X-100) partially purified by ammonium sulfate precipitation. Slides were washed in

PBS and then subjected to avidin-biotin-horseradish peroxidase staining as recommended by the manufacturer (Vector, Burlingame, California) and counterstained in Gill's hematoxylin. Alternatively, sections were stained with hematoxylin and eosin prior to dehydration and mounting in Permount.

FIGURE LEGENDS

Fig.1. *In vitro* infection of breast epithelial cell line HBL-100 and breast tumour cell lines MDA-MB-468, MCF7, MDA-MB-435, T-47D and SK-BR-3. Cells were infected at a multiplicity of infection (MOI) of 10 plaque forming units (PFU) per cell and photomicrographs taken 48 hours post-infection. Cytopathic effect is apparent in all tumor-derived cell lines.

Fig.2. Metabolic radiolabelling of reovirus-infected cells. Cells were infected (MOI=10 PFU/cell) and pulse-labelled with [^{35}S] methionine-containing medium for 4 hours at 26 hours post-infection. Cells were then lysed, and reovirus proteins were immunoprecipitated from part of the lysate using rabbit polyclonal anti-reovirus antibodies and run on SDS-PAGE prior to autoradiography. The three size classes of reovirus proteins (λ, μ, and σ) are indicated on the right. *Top panel*: Whole cell lysate shows reovirus protein synthesis in tumor-derived MDA-MB-468, MCF7, MDA-MB-435, T-47D and SK-BR-3 cells, concurrent with host protein synthesis shutoff. Non-tumorigenic HBL-100 cells did not show any reovirus protein synthesis, nor host protein shutoff. *Bottom panel*: Reovirus protein could only be immunoprecipitated from the tumor-derived cell lines.

Fig.3. Reovirus treatment of orthotopic breast tumor xenografts causes rapid tumor regression. 2 x 10^6 human MDA-MB-435S cancer cells were implanted into the mammary fat pad of SCID/NOD mice. Upon palpable tumor establishment, 1 x 10^7 PFU of reovirus (n = 6, open circles) or UV-inactivated reovirus (n = 6, closed circles) was injected intratumorally. Tumors injected with live virus regress dramatically compared to UV-inactivated virus-treated tumors.

Fig.4. Reovirus-mediated oncolysis of orthotopic tumors. Photos of representative **(a)** UV-inactivated virus-treated, and **(b)** live-virus treated MDA-MB-435S tumors at 28 days post-injection.

Fig.5. Reovirus-mediated *in vivo* oncolysis of tumors remote from the site of injection. SCID mice were implanted subcutaneously and bilaterally with approximately 1 x 10^6 MDA-MB-468 human breast tumor cells. Following the establishment of a palpable mass, a single intratumoral injection of either 1 x 10^7 PFU of live reovirus (open circles) or UV-inactivated virus (closed circles) was injected into the left flank tumor, and tumor growth was followed for a period of four weeks. Tumor regression was observed in both the *injected* (squares) and *remote* (circles) sites in the live-virus treated animals.

Fig.6. Hematoxylin/Eosin staining **(a** and **b)** and immunohistochemistry **(c** and **d)** of reovirus antigens in virus-treated MDA-MB-468 tumors. **(a** and **b)** H/E staining reveals destruction of tumor cells in live virus–treated animals, whereas UV-inactivated virus-treated tumors are intact. **(c)** Periphery of the remaining scar mass from the contralateral, uninjected tumor stains positive for reovirus (brown), where some residual tumor cells remain. **(d)** UV-inactivated virus-treated control tumor exhibits no staining for reovirus protein.

Fig.7. Reovirus replication in primary human tumor tissue samples. A single cell suspension was infected with reovirus and protein synthesis was monitored by incorporation of [^{35}S]-methionine from 24 to 48 hours post-infection. The three size classes of reovirus proteins (λ, μ, and σ) are indicated on the left.

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Fig. 1

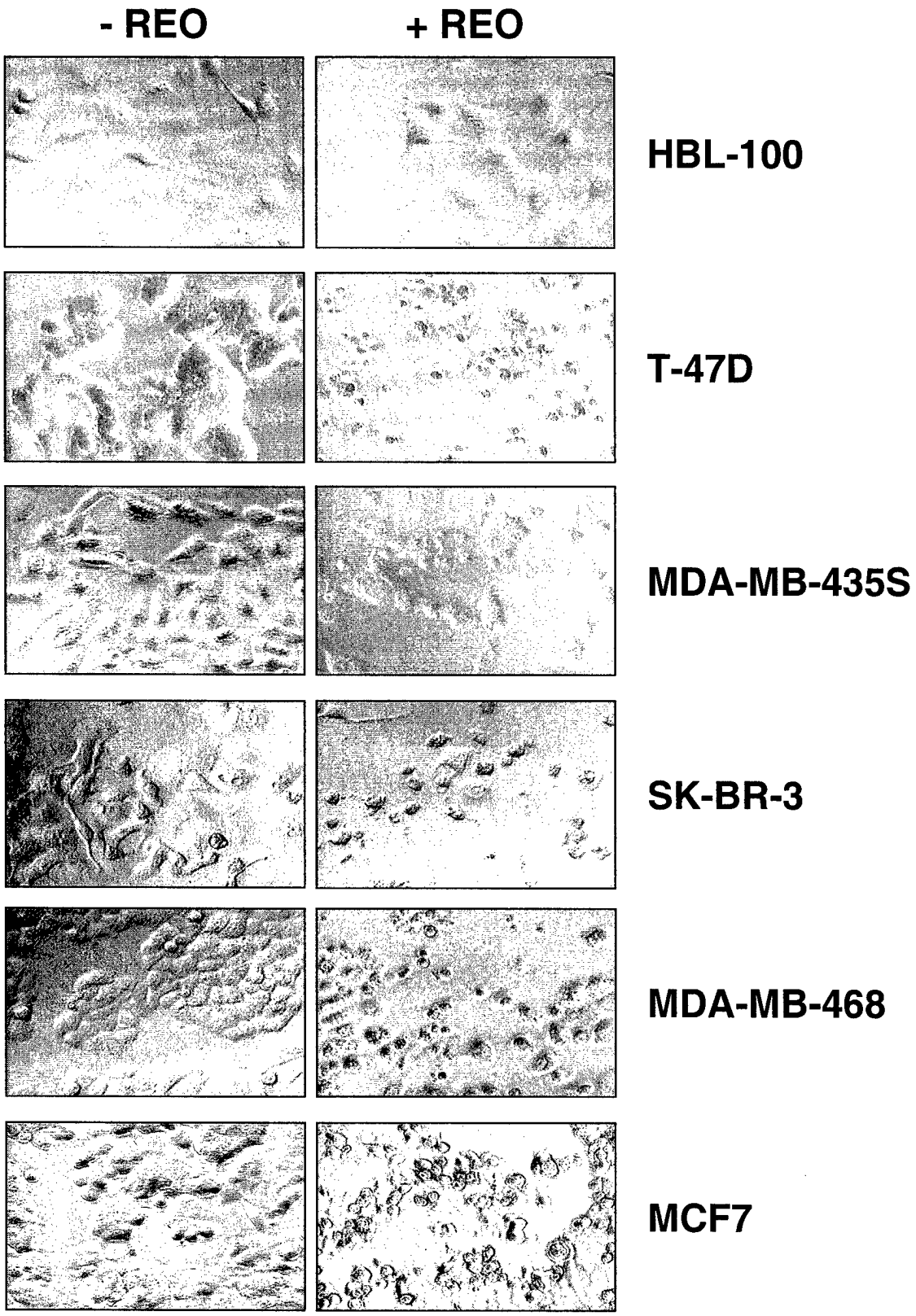
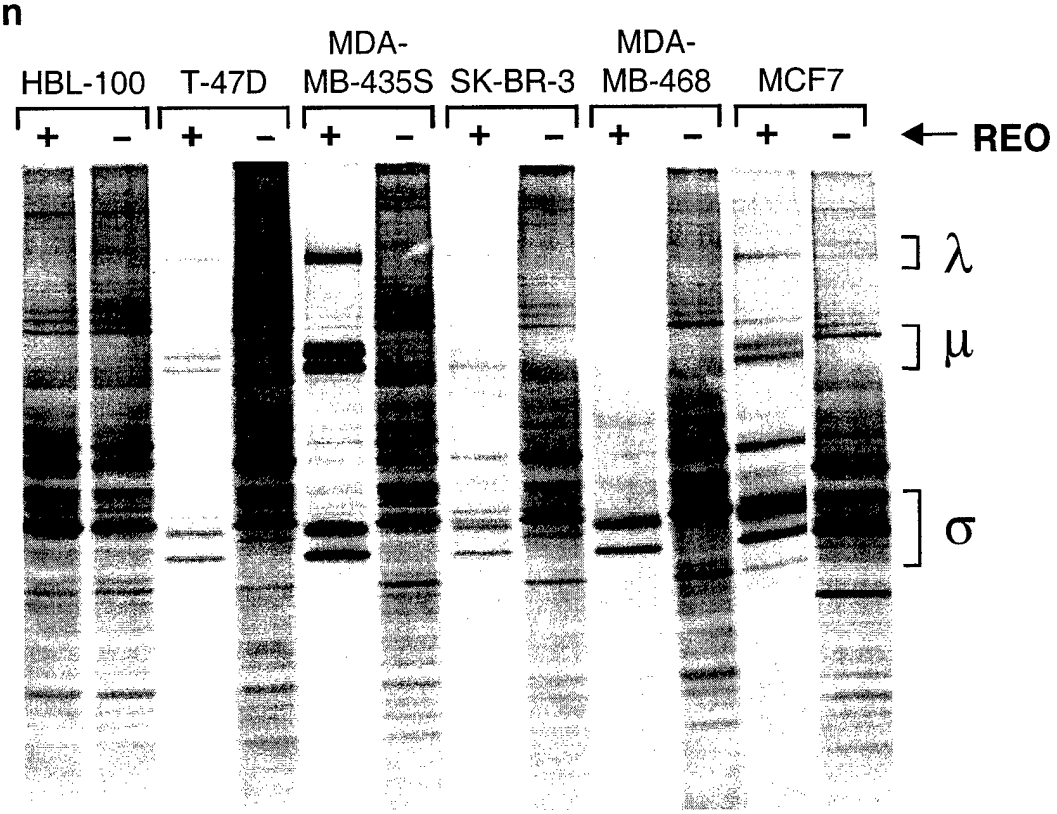


Fig. 2

Expression



Immunoprecipitation

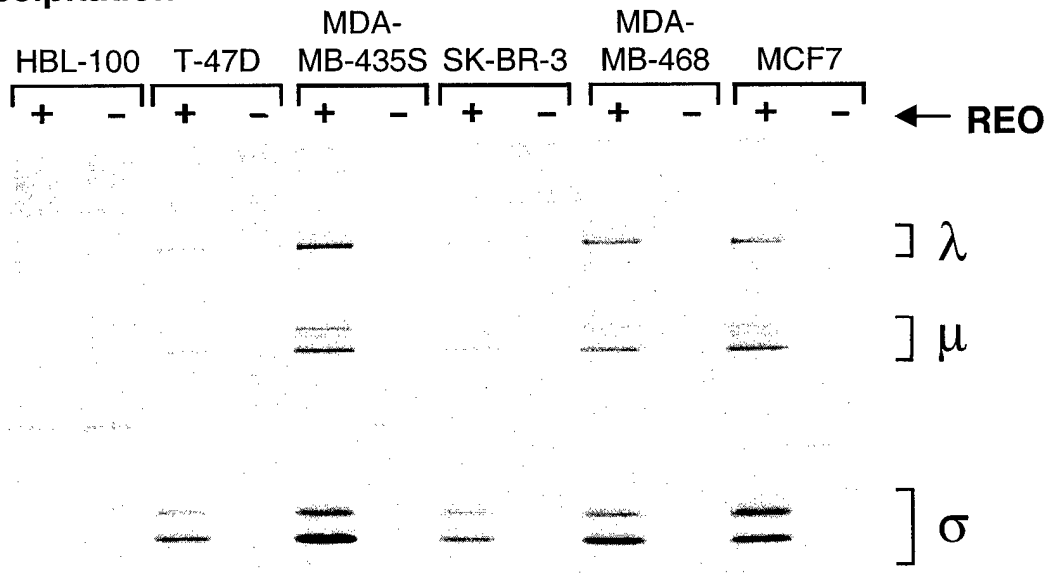


Fig. 3

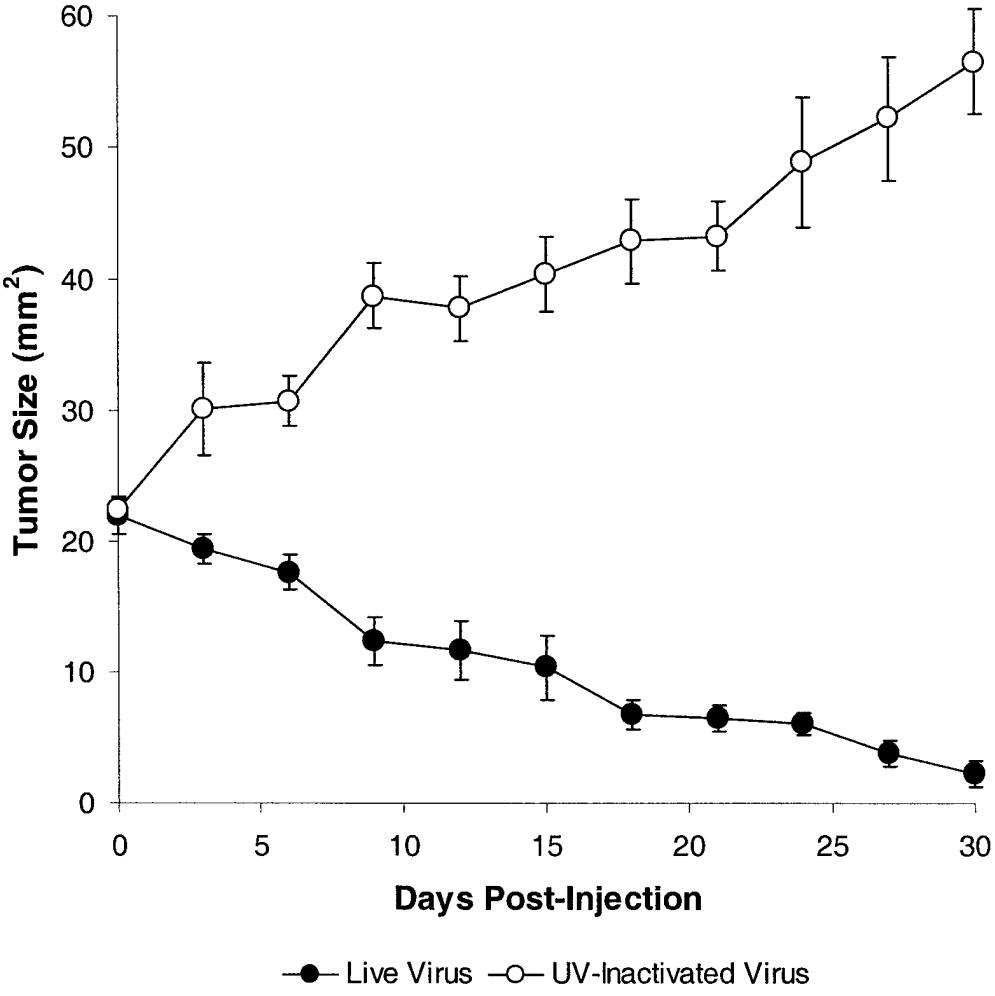
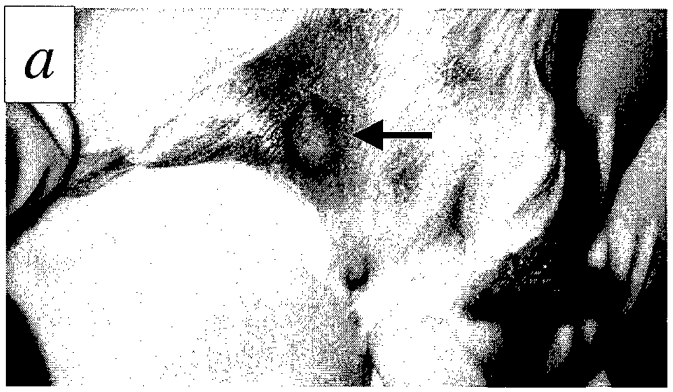
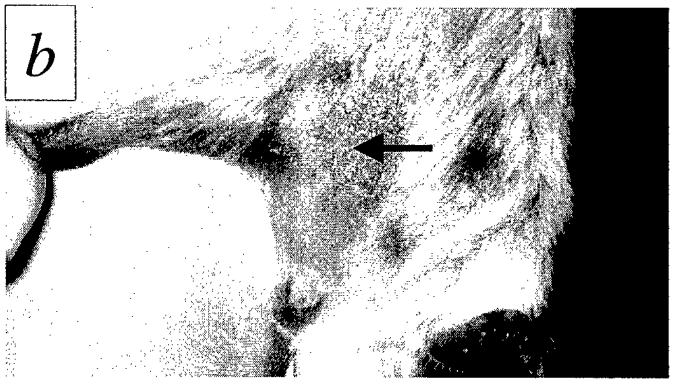

60
50
40
30
20
10
0
Tumor Size (mm^2)
0
5
10
15
20
25
30
Days Post-Injection
Live Virus
UV-Inactivated Virus

Fig. 4



UV-inactivated virus-treated



Live virus-treated

Fig. 5

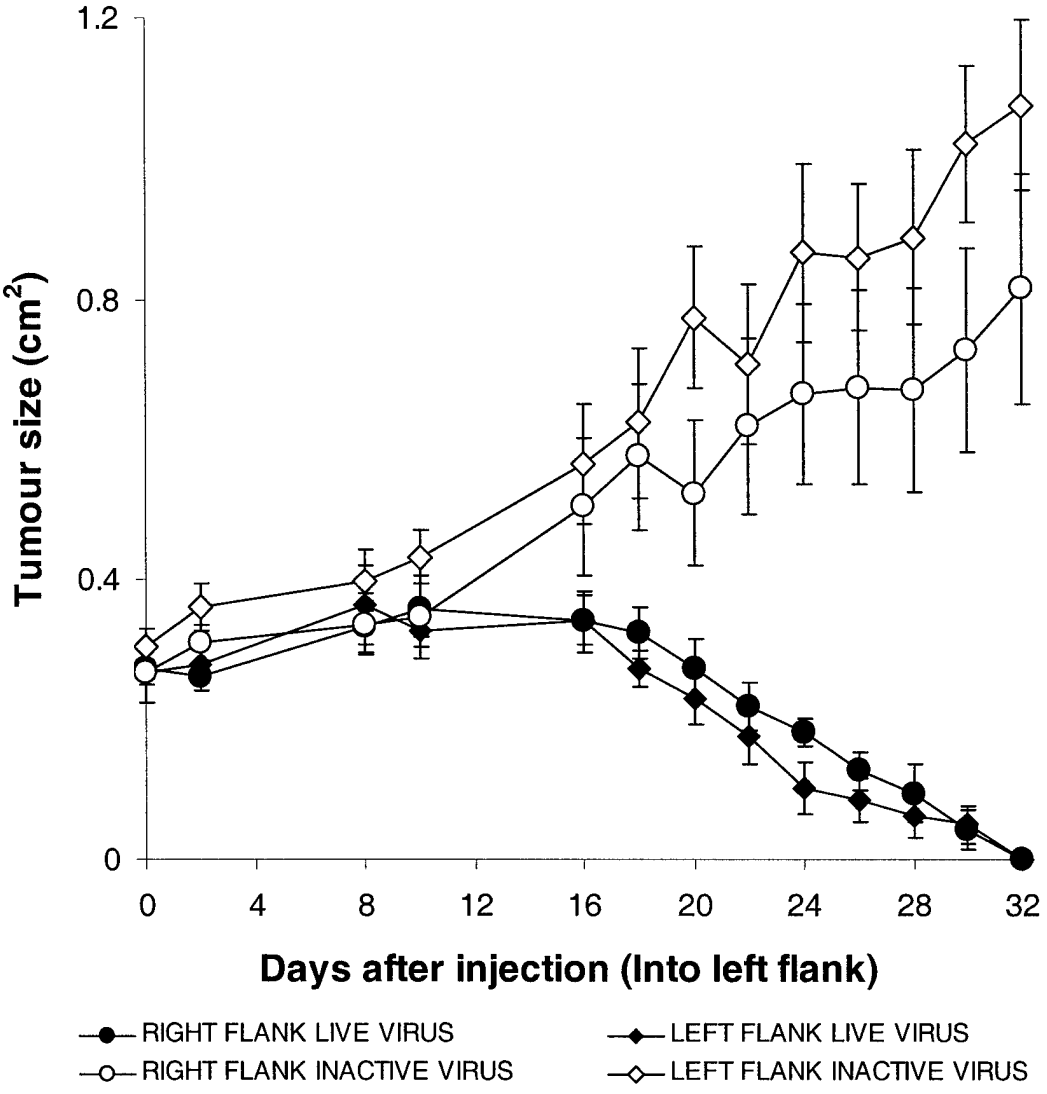
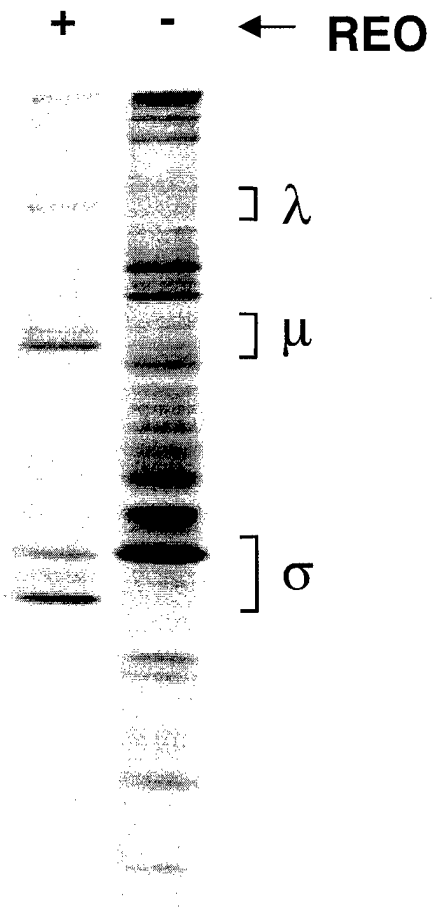

1.2
0.8
0.4
0
Tumour size (cm²)
0
4
8
12
16
20
24
28
32
Days after injection (Into left flank)
RIGHT FLANK LIVE VIRUS
LEFT FLANK LIVE VIRUS
RIGHT FLANK INACTIVE VIRUS
LEFT FLANK INACTIVE VIRUS

Fig. 6

H & E
IHC
a
c
Live virus treated
b
d
UV-inactivated virus treated

Fig. 7



Key research accomplishments:

- reovirus effectively infects and lyses breast cancer cell lines *in vitro*.
- reovirus effectively replicates in *ex vivo* samples of human breast tumor.
- reovirus causes rapid tumor regression in mammary fat pad tumor xenografts in SCID mice.
- Also in SCID mice, systemic spread of the virus causes regression of tumors remote from the primary injection site.

Reportable outcome:

- A manuscript reporting the findings described in this annual report is in preparation and will soon be submitted to a journal.
- Kara Norman, a Ph.D. graduate student under my supervision, was responsible for most of the experiments described in this report. She is expected to graduate in two years.

Conclusion:

Reovirus is effective in killing breast cancer cell lines grown *in vitro* as well as implants *in vivo* in a SCID mouse model. There is also indication that reovirus can replicate in *ex vivo* samples of human breast tumor. These studies should pave the way for clinical trials using reovirus as an anti-breast cancer agent.

References:

All references are listed in the BODY section.

Appendices:

None. All the data are included in the Body Section.